Growth and differentiation of fast and slow muscles in fetal sheep, and the effects of hypophysectomy

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1. Isometric contractile characteristics of fast-twitch (flexor digitorum longus, FDL; medial gastrocnemius, MG) and slow-twitch (soleus) muscles were determined in pentobarbitone-anaesthetized fetal sheep between 90 and 140 days gestation. Five fetuses were hypophysectomized (HPX) at 90–95 days gestation and then studied at 138–140 days.

2. At 90–95 days gestation the time to peak of single twitch contractions for the soleus, MG and FDL were not significantly different from each other; the mean value (±S.E.M.) for all the muscles at this age was 77·6 ± 9·0 ms. At 120–125 days gestation the MG and FDL contracted significantly faster (44·0 ± 0·9 and 40·8 ± 1·8 ms, respectively) than at 90–95 days, and did not change significantly thereafter. In contrast, the soleus muscle contracted more slowly (111·9 ± 6·6 ms) at 138–140 days than at 90–95 days and 120–125 days gestation.

3. Soleus muscle consisted of type I fibres at all gestational ages. There was no significant change with gestational age in the relative numbers of type I and II fibres in the MG and FDL, but in the diaphragm the number of type I fibres increased and the number of type II fibres decreased between 125 and 138 days gestation.

4. HPX abolished the normal increase of soleus weight relative to body weight between 125 and 138 days but did not alter the change of twitch contraction time with age. HPX significantly prolonged twitch time to peak and time to half-relaxation of MG and time to half-relaxation of FDL at 138 days.

5. The maximum rate of rise of the isometric tetanic contraction was unchanged by HPX in all three hindlimb muscles, but fatigue of MG and FDL was increased.

6. The relative proportions of different fibre types in the hindlimb muscles and the diaphragm were unchanged by HPX, but there was a significant decrease in mean areas of type I and II fibres in the FDL and MG of the HPX fetuses.

We have recently shown (Finkelstein, Andrianakis, Luff & Walker, 1992) that muscles such as the diaphragm, medial gastrocnemius (MG), extensor digitorum longus (EDL) and soleus appear to be fully developed by 140 days gestation in sheep (term is approximately 147 days) in terms of both their isometric twitch and tetanic contractile characteristics, and the relative proportions of type I (slow oxidative), type IIA (fast oxidative glycolytic) and type IIB (fast glycolytic) fibres. The twitch contraction times of the MG and EDL had essentially similar values in 138–140 days gestation fetuses, 2- to 30-day-old lambs and in adult sheep. The twitch contraction time of soleus was longer than that of either MG or EDL by the end of pregnancy and showed a further increase 30 days after birth. Therefore, considerable functional differentiation of fast and slow muscle fibres occurs before birth in this species. One of the major aims of this study was to determine when fast-twitch and slow-twitch contractile characteristics appear during the *in utero* development of sheep.

While skeletal muscle fibres are myogenically specified at the embryonic stage of development (Hoh, 1991), both humoral and neural factors are known to exert a profound influence on the physiological, morphological and histochemical properties of the fibres. For instance, in newborn and adult rats and cats, a distinctly different neuronal firing pattern is present in the motor nerves to the fast- and slow-twitch muscles of the hindlimb, and each particular activity pattern is thought to play a role in determining the contractile characteristics of the individual muscles

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(Navarrete & Vrbova, 1983; Pette & Vrbova, 1985). Cross-innervation studies (Buller, Eccles & Eccles, 1960) and chronic stimulation experiments (Salmons & Sreter, 1976), also indicate that the twitch and tetanic contractile characteristics of the fast and slow muscles are determined to a considerable extent by neural activity. However, whether the activity that is present throughout much of fetal life in the sheep (Ruckebusch, Gaugoux & Eghbali, 1977) plays a role in determining the differentiation of the fast and slow phenotype in skeletal muscle is not known.

The contribution of pituitary hormones to fetal somatic growth in general, and to muscle fibre growth and differentiation in particular, remains unclear. Fetal hypophysectomy (HPX) in sheep at approximately 75 or 115 days gestation resulted in reduced body weight and a reduction of limb lengths by approximately 30% (Mesiano, Young, Baxter, Hintz, Browne & Thorburn, 1987; Deayton, Young & Thorburn, 1993). The effects of removing the pituitary on muscle development is not known, but some effects might be expected because of the hypothyroid condition of HPX fetal sheep. We have previously shown that thyroidectomy of fetal sheep resulted in significantly slower twitch contraction times of two fast-twitch muscles (MG and extensor digitorum longus, EDL), but paradoxically, there was an increase in the number of histochemically identifiable fast (type II) muscle fibres in these muscles (Finkelstein, Andrianakis, Luff & Walker, 1991). The interpretation of these results is complicated by the retardation of fetal brain growth and neural maturation which occurs after thyroidectomy (Bhakthavathsalan, Mann, Ayromlooi, Kunzel & Lui, 1977; Walker & Schuijers, 1989), an effect not seen after HPX (Deayton *et al.* 1993). Also, in the presence of thyroid hormone, growth hormone is known to affect muscle growth in postnatal rats (Scow, 1959), but specific effects of these hormones on the development of fast-twitch and slow-twitch fibres have not been examined in this or other species. Therefore, the second aim of this study was to determine if HPX at mid-gestation in fetal sheep had significant effects on the muscle fibre development which normally occurs in the last part of gestation.

METHODS

Animals

Twenty-one pregnant Border Leicester–Merino cross ewes were used in this study. All procedures had been previously approved by the Standing Committee on Ethics in Animal Experimentation of the University.

Sixteen of the ewes were anaesthetized by intravenous injection of pentobarbitone (details given below) at 90–95 ($n = 4$), 100–105 ($n = 4$), 120–125 ($n = 4$) and 138–140 ($n = 4$) days of gestation. Isometric contractions of the fetal hindlimb muscles were studied as described below. In another five ewes the fetal pituitary was removed using sterile procedures under general anaesthesia (halothane, 1·5–2%; $N_2O : O_2$, 50 : 50 v/v) at 90–95 days gestation (Mesiano *et al.* 1987). These ewes were then anaesthetized with pentobarbitone on days 138–140 and the isometric contractions of the hindlimb recorded as for the other four fetal groups. Completeness of the HPX was confirmed at the time of autopsy when the pituitary fossa was examined to reveal complete absence of tissue in all of the five fetuses.

Recording the isometric contractile properties

Anaesthesia of each ewe was induced by an intravenous injection of Pentothal (thiopentone, 1 g in 20 ml water; Boehringer Ingelheim, Artamon, NSW, Australia). The ewe was then intubated, and anaesthesia maintained by intermittent infusion of 3–5 mg kg^{-1} of pentobarbitone sodium through an indwelling catheter placed in the foreleg vein or jugular vein so that only a weak corneal reflex could be elicited. The ewe was then placed on the table on her right side and mechanically ventilated. A catheter was placed in one carotid artery from which blood samples were obtained at intervals for the measurement of blood P_{O_2}, P_{CO_2} and pH using an ABL30 blood gas analyser (Radiometer, Copenhagen). Tidal volume and ventilation rates were adjusted and O_2 added to the inspired air, if necessary, to maintain blood gases and pH in their normal ranges.

The abdomen of the ewe was opened in the mid-line, and the uterine horn containing the fetus was withdrawn onto a table covered by a heated blanket. The fetus was then removed from the uterus through a 6–10 cm incision taking care not to stretch or damage the umbilical cord. The fetus and uterus were covered by a large plastic bag to prevent loss of moisture and heat by evaporation, and also wrapped in the heating blanket and towelling to maintain the fetal temperature, monitored with a probe in the rectum, at 38–39 °C, which is in the range of normal fetal temperatures (Andrianakis, Walker, Ralph & Thorburn, 1989). The right fetal hindlimb was extended and the soleus, MG and FDL muscles were dissected free of surrounding tissues, taking care not to damage the blood and nerve supplies. The hindlimb was then secured with pins inserted into either end of the tibia. The muscles were kept moist with saline at all times during the dissection, and during the experiment were covered with warm mineral oil contained in a pool made by drawing up the skin flaps to a wire frame held above the leg. The oil was maintained at 38–39 °C by heating lamps controlled by a thermistor probe placed in the pool near to the muscles. The distal tendon of each muscle was cut and attached to either a Devices UFI (16 oz; Welwyn Garden City, Herts, UK) dynamometer (for soleus and FDL) or a purpose-built force transducer (for MG). The transducers were calibrated in terms of newtons (N) or millinewtons (mN). Muscle length could be adjusted in 1 mm steps by means of a screw vernier on which the transducer was mounted. The nerve to each muscle was stimulated with pulses of 0·05 ms duration (Devices, model 2533), and supramaximal intensity through a pair of platinum wire electrodes, and the twitch and tetanic contractions were digitized and stored on the hard disk of a microcomputer for later analysis.

The optimum length for isometric peak force for twitch and tetanic contractions was determined for each muscle, and several twitch responses were obtained for the determination of maximum twitch force (N), time to peak (ms) and time to half-relaxation (ms). The force–frequency relationship was determined for each muscle by stimulating with frequencies from 10 to 300 Hz, from which the maximum rate of rise and maximum tetanic force were determined. Finally, an estimate of fatigue was obtained for each muscle by stimulating them once every 1 s for 330 ms and 40 Hz for a period of 800 s. Two fatigue indices were obtained by calculating the ratio of the forces produced during (a) the 1st and 120th tetani, and (b) the 1st and 800th tetani using a modification of the method first described by Burke, Levine, Zajac, Tsairis & Engel (1971).

The experiments under barbiturate anaesthesia, during which isometric twitch and tetanic contractions were measured, took

Table 1. Fetal body and hindlimb muscle wet weights from 90 to 140 days gestation in intact fetal sheep and hypophysectomized (HPX) fetuses at 138–140 days gestation for each group

			Soleus		MG		FDL	
Gestational age (days)	n	Fetal wt (g)	Wt (g)	Wt/body wt ($\times 10^{-3}$ %)	Wt (g)	Wt/body wt ($\times 10^{-3}$ %)	Wt (g)	Wt/body wt ($\times 10^{-3}$ %)
Intact								
90–95	4	647 ± 21	0·059 ± 0·006	9·2 ± 1·3	0·79 ± 0·06	123 ± 8	0·11 ± 0·01	16·7 ± 1·8
100–105	4	1385 ± 160	0·130 ± 0·009	9·4 ± 1·1	1·54 ± 0·05	111 ± 14	0·15 ± 0·01	10·8 ± 1·4
120–125	4	2554 ± 44	0·222 ± 0·027	8·7 ± 1·4	3·34 ± 0·17	131 ± 7	0·41 ± 0·01	16·1 ± 1·5
138–140	4	4032 ± 276	0·839 ± 0·173	20·8 ± 3·21*	6·07 ± 0·28	151 ± 8	0·84 ± 0·08	20·8 ± 4·2
HPX								
138–140	5	3052 ± 108†	0·243 ± 0·033†	8·0 ± 1·0†	3·23 ± 0·33†	109 ± 11	0·49 ± 0·02†	17·0 ± 1·0

* $P < 0·05$, 120–125 *vs.* 138–140 days; † $P < 0·05$, HPX *vs.* intact fetuses at 138–140 days.

between 3 and 4 h to complete. A blood sample obtained by puncture of the fetal umbilical artery at the end of the experiments showed that fetal blood gases (P_{O_2}, 20·2 ± 1·4 mmHg; P_{CO_2}, 47·5 ± 1·8 mmHg), pH (7·388 ± 0·019), O_2 saturation (56·5 ± 5·0) and haemoglobin concentration (12·4 ± 0·3 g%) (means ± S.E.M.) were in the normal range (Dawes, Fox, Leduc, Liggins & Richards, 1972).

At the end of these procedures the ewe and fetus were killed by an overdose of pentobarbitone (130 mg (kg ewe)$^{-1}$), and the three fetal limb muscles and a costal strip of diaphragm were immediately removed, weighed, set at their *in vivo* lengths using an adjustable caliper, and frozen in 2-methyl butane cooled in liquid N_2. The muscles were then wrapped in aluminium foil and plastic film and stored at −70 °C until used for histochemical analysis of fibre types.

Histochemistry

Serial sections of each muscle, 10–12 μm thick, were cut on a freezing cryostat microtome and adjacent sections were processed using either (a) the myosin adenosinetriphosphatase (mATPase) reaction at pH 4·3 (Guth & Samaha, 1970) or (b) the NADH-tetrazolium reductase method (Novikoff, Woo-Yung & Drucker, 1961). Using results from both reactions muscle fibres were classified where possible and depending on gestational age as slow oxidative (or type I), fast oxidative glycolytic (or type II A) and fast glycolytic (or type II B) according to the criteria of Peter, Barnard, Edgerton, Gillespie & Stempel (1972). Representative fields of each section were projected at ×330 magnification onto a Zeiss-MOP graphics tablet via a front-silvered mirror. A digitizing pen was then used to determine the area within the perimeter traced around each fibre type and the results stored on computer disk for later analysis of the mean number and area of each fibre type in each muscle, using a spreadsheet program.

Statistics

All data are presented as means ± S.E.M. Differences between measured parameters were determined by three-way ANOVA using animal, gestational age and muscle as the factors. When a significant interaction between factors was identified, a Student–Newman–Keuls test was applied *post hoc*, with $P < 0·05$ selected as the level of significance. Differences between parameters measured in the intact and HPX fetuses at 138–140 days gestation were compared using Student's unpaired t test. When necessary, the data were transformed to equalize the variances of the groups before ANOVA was applied, and in all cases the use of a parametric test was then appropriate.

RESULTS

The fetal body and muscle weights at each gestational age are shown in Table 1. The weight of all three hindlimb muscles increased with gestation, but relative to body weight the soleus muscle increased significantly between 125 and 138 days gestation from 0·0087 to 0·0208% of body weight (Table 1). HPX resulted in a significant decrease of fetal body weight and absolute weights of the three muscles. However, the soleus alone was reduced in weight relative to the fetal body weight.

Isometric contractions

Gestational age. Representative records of single twitch contractions for each of the three hindlimb muscles at each gestational age are shown in Fig. 1. As expected, peak twitch force increased with increasing gestational age. To facilitate comparison of the contraction and relaxation phases of the twitches obtained at each gestational age, the twitch responses were normalized by replotting each of them in relation to their peak force, and then superimposing the traces for each muscle at the different gestational ages (Fig. 2). At 90 days gestation the contraction and relaxation times were similar for the three hindlimb muscles. There was a significant decrease of the twitch times for the MG and FDL between 105 and 125 days gestation, whereas no significant change occurred over this time for the soleus muscle. At both 105 and 125 days gestation the MG and FDL contracted and relaxed significantly faster than the soleus, but they did not differ from each other (Figs 2 and 3). The twitch contraction and relaxation times of the MG and FDL did not change further between 125 and 140 days gestation, whereas the twitch contraction time of the soleus muscle became significantly slower between these two gestational ages (Fig. 3).

The forces generated during twitch and tetanic contractions increased with age for each muscle when expressed in absolute units (N; Table 2). There was no consistent change of the ratio of peak twitch to peak tetanic force with gestational age for the MG and FDL, although it increased slightly, but not significantly, for the soleus. The maximum

Table 2. Isometric twitch and tetanic contraction parameters of intact fetal hindlimb muscles at 90–140 days of gestation and HPX fetuses at 138–140 days of gestation

Gestational age (days)		Peak force (N)		Relative peak force (N (g wet wt)$^{-1}$)		Max rate of rise † (%)	Twitch/tetanic force ratio
		Twitch	Tetanus	Twitch	Tetanus		
Soleus							
90–92	4	0·010 ± 0·003^{a}	0·040 ± 0·007^{a}	0·195 ± 0·063	0·724 ± 0·157	2·69 ± 0·26^{a}	0·23 ± 0·04^{a}
100–105	4	0·017 ± 0·004^{a}	0·066 ± 0·015^{a}	0·129 ± 0·028	0·506 ± 0·098	2·57 ± 0·44^{ab}	0·25 ± 0·03^{b}
120–125	4	0·067 ± 0·008^{b}	0·244 ± 0·032^{ab}	0·334 ± 0·068	1·227 ± 0·272	2·26 ± 0·35^{b}	0·27 ± 0·01^{a}
138–140							
Intact	4	0·144 ± 0·055^{c}	0·513 ± 0·178^{b}	0·199 ± 0·078	0·729 ± 0·258	2·16 ± 0·25^{ab}	0·28 ± 0·03^{a}
HPX	5	0·078 ± 0·016	0·330 ± 0·063	0·472 ± 0·010*	2·101 ± 0·562	1·55 ± 0·01	0·24 ± 0·04
MG							
90–92	4	1·06 ± 0·09^{a}	1·97 ± 0·15^{a}	1·62 ± 0·49	2·53 ± 0·59	1·66 ± 0·14^{a}	0·55 ± 0·06^{a}
100–105	4	1·89 ± 0·36^{a}	2·89 ± 0·55^{a}	2·30 ± 0·18	3·52 ± 0·27	1·89 ± 0·08^{ab}	0·65 ± 0·05^{b}
120–125	4	4·43 ± 0·34^{b}	10·91 ± 0·033^{b}	1·27 ± 0·47	2·49 ± 0·87	3·16 ± 0·20^{b}	0·40 ± 0·02^{a}
138–140							
Intact	4	10·04 ± 1·04^{c}	26·24 ± 1·93^{c}	1·65 ± 0·13	4·35 ± 0·38	2·57 ± 0·05^{ab}	0·40 ± 0·07^{a}
HPX	5	5·93 ± 1·77	10·38 ± 3·18*	2·03 ± 0·13	4·97 ± 1·27	2·35 ± 0·11	0·41 ± 0·06
FDL							
90–92	4	0·18 ± 0·07^{a}	0·28 ± 0·09^{a}	1·34 ± 0·12	2·56 ± 0·34	2·45 ± 0·27^{a}	0·59 ± 0·05^{a}
100–105	4	0·35 ± 0·02^{a}	0·55 ± 0·41^{b}	1·20 ± 0·19	1·84 ± 0·28	2·80 ± 0·41^{ab}	0·65 ± 0·04^{b}
120–125	4	0·56 ± 0·22^{b}	1·08 ± 0·41^{b}	1·32 ± 0·04	3·28 ± 0·07	3·33 ± 0·25^{b}	0·46 ± 0·06^{a}
138–140							
Intact	4	1·30 ± 0·47^{c}	3·29 ± 0·99^{c}	1·64 ± 0·65	4·14 ± 1·42	3·55 ± 0·60^{ab}	0·36 ± 0·04^{a}
HPX	5	0·42 ± 0·14	0·71 ± 0·21*	0·93 ± 0·36	1·56 ± 0·56	2·90 ± 0·22	0·59 ± 0·06

† Percentage of peak tetanic force. Superscripts, where different, indicate significant differences between ages for each muscle. *$P < 0·05$, HPX *vs.* intact fetuses at 138–140 days.

rate of rise of force (MRR) during tetanic contractions was significantly higher in the FDL compared with the MG at all gestational ages. However, MRR did not change significantly with age for any of the muscles (Table 2).

Two indices of fatigue were calculated as the ratio of peak force developed during the 1st and 120th tetani (F_1), and during the 1st and 800th tetani (F_2) with each tetanus being produced by stimulating the muscle at 40 Hz for 330 ms, once every 1 s (Burke *et al.* 1971). Because force decreases with repeated tetani, indices of fatigue are always < 1 and become smaller as fatigue increases. In all three hindlimb muscles the fatigue indices increased significantly between 105 and 125 days gestation, but there was no difference between any of the three muscles at any gestational age (Table 3).

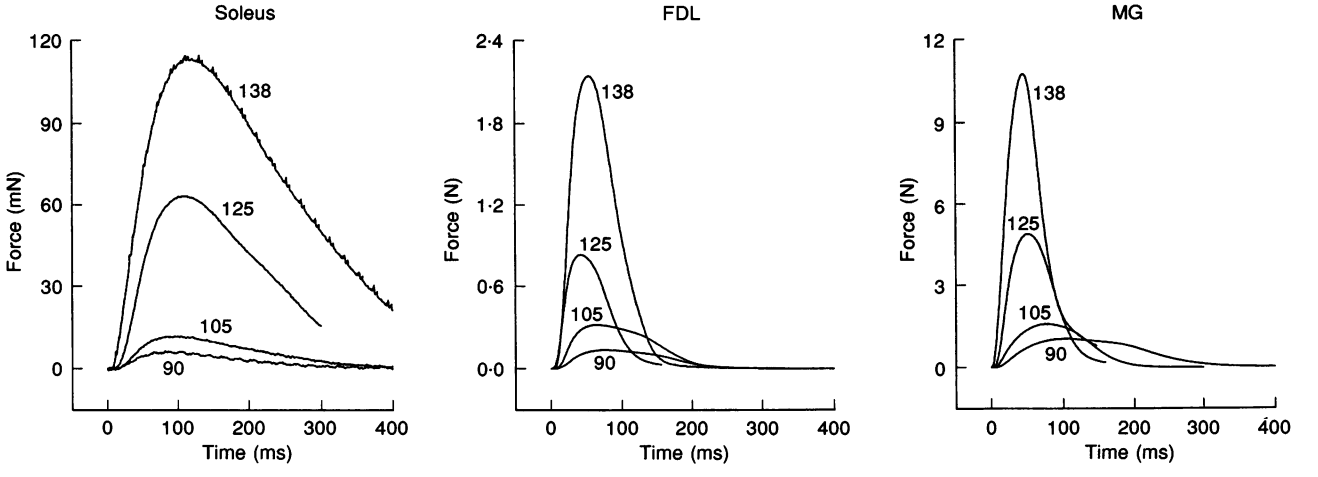

Figure 1

Representative twitch contractions for the soleus, MG and FDL at the four gestational ages (days) indicated next to the curves.

Table 3. Fatigue indices for intact fetal hindlimb muscles at 90–140 days gestation and HPX fetuses at 138–140 days gestation

Gestational age (days)	n	Fatigue index F_1	Fatigue index F_2
Soleus			
90–92	4	0·69 ± 0·07[a]	0·49 ± 0·08[a]
100–105	4	0·58 ± 0·05[a]	0·37 ± 0·07[a]
120–125	4	0·70 ± 0·06[b]	0·58 ± 0·08[b]
138–140			
Intact	4	0·72 ± 0·08[b]	0·64 ± 0·05[b]
HPX	4	0·58 ± 0·05	0·48 ± 0·07
MG			
90–92	4	0·50 ± 0·04[a]	0·32 ± 0·13[a]
100–105	4	0·59 ± 0·02[a]	0·47 ± 0·01[a]
120–125	4	0·76 ± 0·04[b]	0·58 ± 0·03[b]
138–140			
Intact	4	0·74 ± 0·03[b]	0·59 ± 0·03[b]
HPX	4	0·53 ± 0·05*	0·33 ± 0·04*
FDL			
90–92	4	0·65 ± 0·08[a]	0·49 ± 0·07[a]
100–105	4	0·64 ± 0·07[a]	0·51 ± 0·02[a]
120–125	4	0·81 ± 0·12[b]	0·49 ± 0·05[a]
138–140			
Intact	4	0·81 ± 0·02[b]	0·60 ± 0·05[b]
HPX	3	0·70 ± 0·04*	0·37 ± 0·07*

See Methods for definition of F_1 and F_2. Superscripts, where different, indicate significant differences between ages for each muscle. * $P < 0·05$, HPX *vs.* intact fetuses at 138–140 days.

Effect of HPX

The twitch time to peak was significantly longer for the MG of the HPX fetuses and twitch time to half-relaxation was significantly longer for both the MG and FDL of the HPX fetuses compared with control fetuses (Table 4). The twitch time to peak for the intact FDL was longer than for the HPX fetus, but this was not quite significant ($P = 0·06$). In contrast, the twitch times to peak and half-relaxation of the soleus muscle of intact and HPX fetuses were not different.

The absolute peak force was decreased slightly, but not significantly, in all three muscles in the HPX fetuses compared with intact fetuses at 138–140 days gestation (Table 2). Peak tetanic force was significantly less for MG and FDL. Whereas the peak twitch and tetanic forces

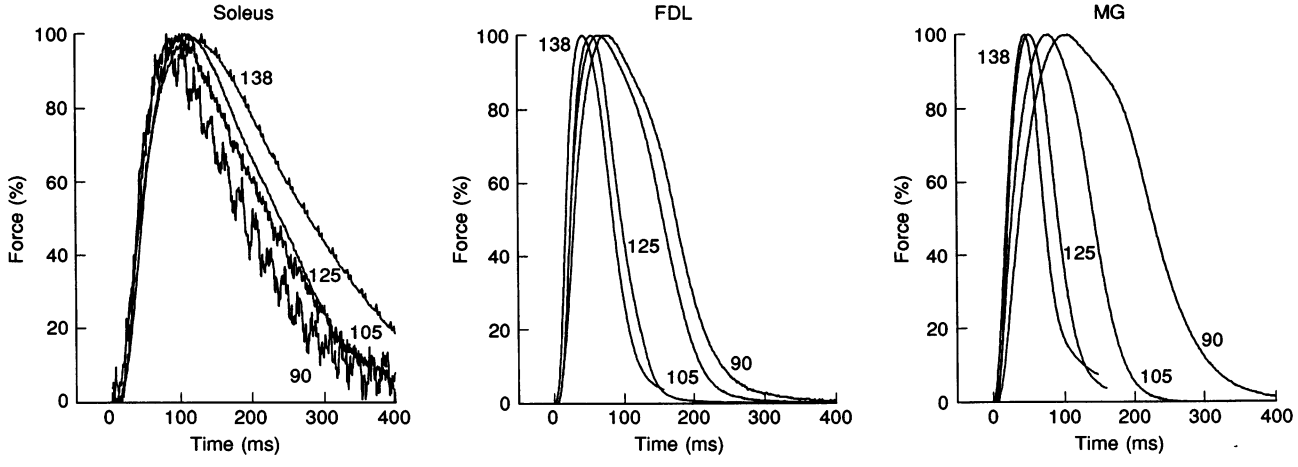

Figure 2

Twitch contractions for the soleus, MG and FDL at the four gestational ages (days) indicated next to the curves. Each of the records shown in Fig. 1 have been re-scaled using the peak force of each contraction as 100%.

Table 4. Time to peak and time to half-relaxation for isometric twitch contractions of soleus, MG and FDL of intact and HPX fetuses at 138 days gestation

	n	Time to peak (ms)	Time to half-relaxation (ms)
Soleus			
Intact	4	111·7 ± 4·0	129·5 ± 27·0
HPX	5	113·9 ± 3·6	112·8 ± 9·5
MG			
Intact	4	41·0 ± 1·4	29·1 ± 1·0
HPX	5	57·5 ± 2·6*	52·5 ± 5·6*
FDL			
Intact	4	44·2 ± 2·8	39·6 ± 3·2
HPX	5	52·0 ± 2·2	62·6 ± 7·9*

*$P < 0·05$ *vs.* intact fetuses.

relative to muscle mass were not significantly changed in MG and FDL, the relative peak twitch force was increased significantly in the soleus muscle. There was no significant change in the maximum rate of rise of force during a tetanus in all three muscles after HPX (Table 2). HPX resulted in significant decreases in both indices of fatigue in MG and FDL (Table 3). There was also a reduction of the fatigue indices in the soleus muscle, but this was not significant (F_1, $P = 0·08$; F_2, $P = 0·11$).

Fibre and morphometric analysis

Gestational age. Between 200 and 500 fibres were measured in each muscle at each gestational age in the intact fetuses and in the muscles from the HPX fetuses. Muscles from between three and five fetuses were obtained for each group and the results averaged. Using the mATPase reaction after pre-incubation of the sample at pH 4·3 it was possible to distinguish two populations of fibres at all gestational ages; i.e. dark- and light-staining fibres, which were designated as type I and type II fibres, respectively. At 138–140 days gestation two groups of fibres could also be distinguished as NADH+ and NADH− on the basis of a difference in density of reaction product granules, but at earlier gestational ages this distinction could not be made. Therefore, attempts to classify fibres further as oxidative and glycolytic were not attempted. Also, reversal of the mATPase reaction by pre-incubation of the sample at pH 10·6 did not occur unequivocally in fetal muscles prior to 138–140 days, and it was not possible to distinguish type II A and II B fibres using this procedure. Thus, although three fibre types could be identified at 138–140 days gestation as we have shown previously (Finkelstein *et al.* 1992), fibres have been classified only as types I and II on the basis of the results obtained using the mATPase reaction at pH 4·3.

The soleus muscle consisted entirely of type I fibres at all gestational ages. Type I and II fibres were identifiable in the MG, FDL and diaphragm from 90 days gestation. Approximately 10–20% of the fibres in MG and FDL were type I, and there was no significant change in the relative numbers of type I and II fibres in these muscles with gestational age. In the diaphragm the number of type I fibres increased significantly from 16·2 ± 1·1 to 24·2 ± 2·8% ($P < 0·05$) between 125 and 140 days gestation. The fibres of the MG, FDL and diaphragm increased 2- to 4-fold in size between 90 and 140 days gestation at an approximately steady rate (Fig. 4). However, in the soleus muscle, fibre size increased from 55·9 ± 3·4 to 567·1 ± 68·7 μm^2 over this time, with an approximate 3-fold increase in size in late gestation between the 120–125 and 138–140 day age groups (Fig. 4).

HPX

The relative proportions of type I and II fibres in the three hindlimb muscles were not different in the HPX compared with the intact fetuses at 138 days. In the diaphragm there

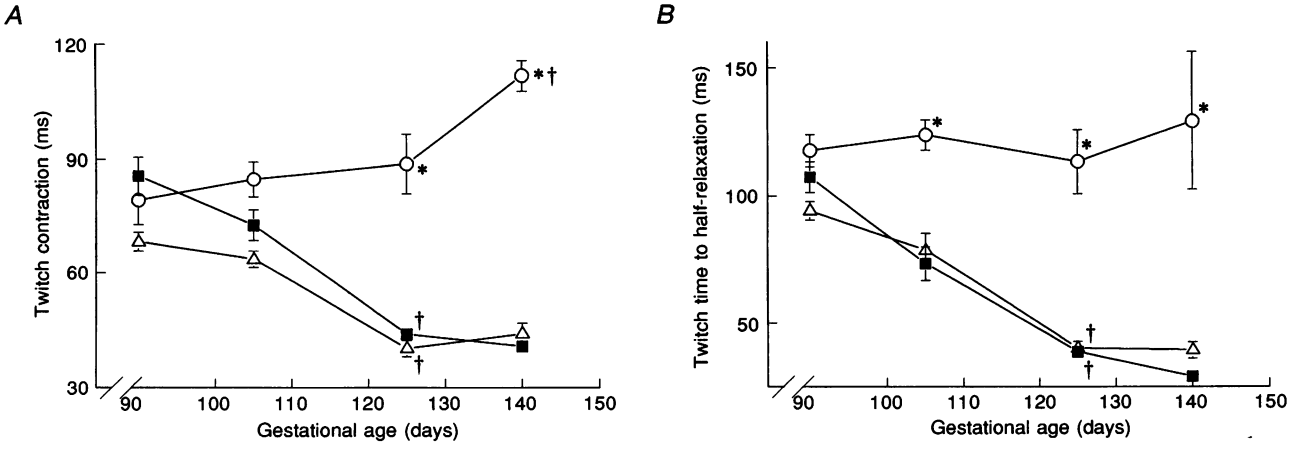

Figure 3

Time to peak (*A*) and time for half-relaxation (*B*) of twitch contractions for the soleus (○), MG (■) and FDL (△) at the four gestational ages shown. * Significant differences between muscles at each gestational age. † Significant differences between ages for each muscle ($n = 4$ throughout).

was a signficant reduction in the proportion of type I fibres from 24·3 ± 3·4 to 10·3 ± 2·5% ($P < 0·05$). The mean fibre area of type I and II fibres was significantly reduced in the FDL and the MG of HPX fetuses (Fig. 5). The mean size of fibres in the soleus was also reduced, but not significantly. There was no significant change in the mean size of fibres in the diaphragm after HPX although type II fibres tended to be smaller (Fig. 5).

DISCUSSION

In this study we have shown that the twitch contraction and half-relaxation times for three hindlimb muscles, which are typically slow-twitch (soleus) and fast-twitch (MG and FDL) muscles in the adult, were not significantly different from each other at 90 days gestation. With further development there was a significant decrease in the twitch contraction times of the MG and FDL, and a prolongation of the twitch duration in the soleus. Importantly, these changes occurred in the slow- and fast-twitch muscles at different times in development. There was a significant decrease of the twitch contraction times for the MG and FDL between 90 and 125 days gestation, with no further change until the end of gestation. In contrast, the increase in twitch contraction time in the soleus muscle occurred only after 125 days of gestation.

The different gestational ages at which these changes occurred, and the fact that they were opposite in direction for the fast- and slow-twitch muscles, suggests that different regulatory mechanisms might be involved for each type of muscle. HPX at 90 days did not completely prevent the development of fast- and slow-twitch characteristics of these hindlimb muscles, and it may be that neurally derived trophic agents, or neural activity itself, is primarily responsible for the functional differentiation of these fast- and slow-twitch muscles. Also, there may be further postnatal changes in all slow-twitch muscle fibres, because in a previous study we showed that the twitch contraction time of the soleus muscle of sheep continued to increase after birth (Finkelstein *et al.* 1992). In contrast, the twitch contraction and half-relaxation times of the MG and FDL attained by the end of gestation were not different from those found in newborn lambs and adult sheep.

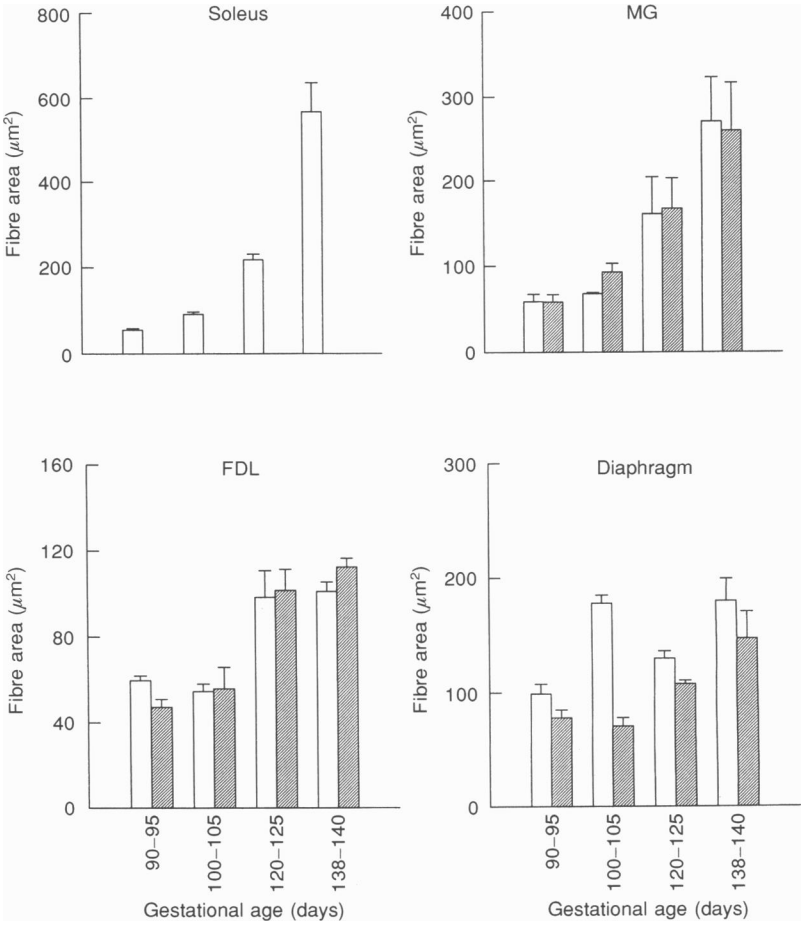

Figure 4

Cross-sectional areas (μm^2) of type I (□) and type II (▨) fibres in the soleus, MG, FDL and diaphragm of fetal sheep at each gestational age range. Values shown are means (±S.E.M.) of 3–5 muscles of each type. Between 200 and 500 fibres were measured in each muscle sample.

Although significant changes occurred in the isometric contractile parameters of the three hindlimb muscles over the last third of gestation, the relative proportions of fast- and slow-twitch fibres did not change during this time. In cats, there is a postnatal decrease in the contraction time of the isolated diaphragm–phrenic nerve preparation, which is not accompanied by changes in the proportions of histochemically identified fast and slow muscle fibres (Sieck & Fournier, 1991). These observations suggest that maturational changes in the isometric twitch contraction time can take place independently of the activities of myosin and mitochondrial enzymes, which have been used conventionally to classify fibre types. It is possible that the development of the sarcoplasmic reticular network and the mechanisms that release and re-sequester calcium play a major role in determining the ontogenetic change in twitch contraction time of skeletal muscle (Luff & Attwood, 1971).

Type II fibres could be differentiated into IIA and IIB populations at 138–140 days gestation, confirming our previous study (Finkelstein *et al.* 1992), but this could not be done convincingly at earlier gestational ages, and we have therefore restricted the classification of fibre types to types I and II on the basis of their mATPase reaction at pH 4·3. On this basis, both 'slow' (type I) and 'fast' (type II) fibres could be identified in the three hindlimb muscles and the diaphragm from at least 90 days of gestation. Type I fibres constituted between 10 and 20% of the fibres at all fetal ages in the MG, FDL and diaphragm, the only significant change being in the diaphragm, where there was a small increase in the relative number of type I fibres and a decrease of type II fibres between 125 and 140 days of gestation. The increase in the proportion of type I fibres in the diaphragm is interesting because this muscle, unlike those of the leg, is rhythmically active throughout at least the latter half of gestation (Dawes *et al.* 1972; Clewlow, Dawes, Johnston & Walker, 1983; Cooke & Berger, 1990). Fetal breathing movements result in a small tidal movement of fluid in the lungs and trachea (Dawes *et al.* 1972) and are important for development of the alveolar space (Harding, Hooper & Han, 1993). Towards the end of gestation breathing movements account for approximately 30% of the resting oxygen consumption of the fetus (Rurak & Gruber, 1983). The role of this activity in influencing the growth and differentiation of fibre types in the diaphragm is

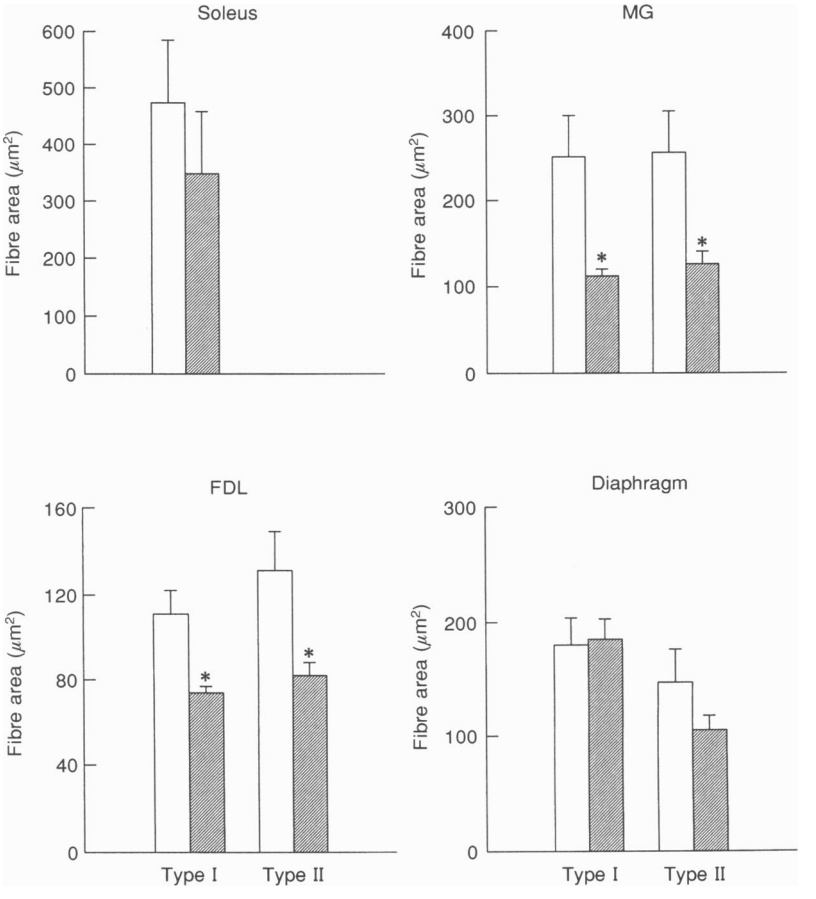

Figure 5

Mean (± S.E.M.) cross-sectional areas (μm^2) of type I and type II fibres in intact (□) and HPX (▨) fetal sheep at 138–140 days gestation. * Significant differences ($P < 0{\cdot}05$; unpaired t test) between groups. $n = 4$ for soleus, MG and FDL; $n = 3$ for diaphragm. Between 200 and 500 fibres were counted in each muscle sample.

unknown, but the increase in the relative number of type I fibres may be important because it is the 'slow' motor units which are recruited during breathing, at both normal and augmented levels of ventilation, in the adult (Sieck, 1992).

HPX resulted in a significant reduction of fetal body weight at 138 days gestation, which is consistent with the findings of two previous studies from this laboratory (Mesiano *et al.* 1987; Deayton *et al.* 1993). The weights of the MG and FDL were reduced proportionately with the reduction of body size, whereas the soleus muscle was disproportionately smaller than body size. The soleus muscle, unlike the MG and FDL, grew significantly faster than the fetus as a whole between 125 and 140 days, and this increase of the soleus muscle and the size of its fibres at the end of gestation thus appears to be a hypertrophic response to a growth factor under the control of the pituitary gland. However, this growth response does not appear to involve all type I fibres because the size of these fibres in the other muscles did not increase in the intact fetuses over the same period of gestation, nor were they selectively reduced in size in the MG, FDL and diaphragm after HPX. The effect of HPX on the soleus muscle could be the result of removing a pituitary factor necessary for the growth of a selected population of slow-twitch muscle fibres, including those in the soleus muscle. Alternatively the effect could have arisen as a consequence of changes in CNS and neuromuscular activity which followed HPX. However, the role of fetal activity in determining muscle phenotype in this species has not yet been elucidated.

HPX was considered to be complete based on the significant reduction of adrenal size and the absence of tissue in the pituitary fossa when the fetuses were examined at autopsy. Adrenal weights of the HPX fetuses ($0{\cdot}274 \pm 0{\cdot}007$ g or $(9{\cdot}026 \pm 0{\cdot}257) \times 10^{-5}$ % of body weight) were significantly less than for sham-operated fetuses at 147 days (Deayton *et al.* 1993). We did not measure adrenal weights in the intact fetuses. Although previous studies have shown that plasma thyroxine (T_4) concentrations are undetectable (i.e. < 2 pg ml^{-1}) 20–40 days after HPX (Wrutniak, Cabello & Bosc, 1986), it has also been noted that fetal plasma free-T_4 concentrations decrease slowly after removal of the pituitary (Wrutniak *et al.* 1986). Thus, the less marked effects of HPX on the two fast-twitch muscles compared with thyroidectomy (Finkelstein *et al.* 1991) may reflect the presence of significant, though decreasing, concentrations of free-T_4 in the fetal circulation for some time after the removal of the pituitary gland at 90 days gestation. Also, it is possible that in the absence of the severe growth retardation of the CNS, which is characteristic of thyroidectomy (Bhakthavathsalan *et al.* 1977), but not HPX (Mesiano *et al.* 1987; Deayton *et al.* 1993), the fast-twitch muscles are able to continue to develop, in part, in the presence of low thyroid hormone concentrations.

HPX did not change the twitch or relaxation times of the soleus, whereas the twitch contraction time of the MG and the time for half-relaxation of both the MG and FDL were increased. The twitch contraction time for the FDL was also increased, but not quite significantly ($P = 0{\cdot}059$). However, both the MG and the FDL in HPX fetuses contracted significantly faster than the same muscles from control animals at 90 and 105 days gestation, indicating that considerable maturation had occurred despite the absence of the pituitary gland. HPX did not change the relative proportions of fibre types in the MG and FDL, indicating that the ATPase enzyme activity is not critically dependent on hormonal regulation by the pituitary–thyroid or the pituitary–adrenal axes. The changes of the twitch contraction profile which occurred in these muscles could thus be related to changes in either isomyosin composition, or to the rate of release and reuptake of calcium from the sarcoplasmic reticulum.

Thyroid hormone alterations are known to produce significant changes in muscle contractile properties in adult rats. These result from changes in fibre-type composition, and activities of myosin ATPase and sarcoplasmic reticulum Ca^{2+}-ATPase (Fitts, Winder, Brooke & Kaiser, 1980; Nwoye, Mommaerts, Simpson, Seraydarian & Marusich, 1982). The prolongation of twitch contraction time of MG and FDL may be due to the virtually athyroid condition of the HPX fetuses (Wrutniak *et al.* 1986; Mesiano *et al.* 1987). Thus, the longer twitch contraction times of the MG and FDL might be the result of decreased activity of the Ca^{2+}-ATPase of the sarcoplasmic reticulum, or retardation of the transition from fetal to neonatal and adult fast myosin isoforms. However, it is of note that fetal thyroidectomy produced somewhat greater increases in the twitch contractions of the MG and another fast-twitch muscle, the extensor digitorum longus (Finkelstein *et al.* 1991). This suggests that the very low plasma T_4 concentrations which are present after HPX (Wrutniak *et al.* 1986) may be sufficient for some maturation of the contractile apparatus in this class of muscle.

The maximum rate of rise (MRR) of isometric tension during a tetanic contraction is dependent upon actin–myosin interactions: specifically, the rate of cross-bridge formation (Lewis, Al-Amood & Rosendorff, 1986). Changes in this parameter of the isometric tetanus therefore reflect alterations which occur in actomyosin ATPase activity. After HPX the MRR was unchanged despite the increase in the twitch contraction times of the FDL and MG. This suggests that a major effect of HPX on developing fast-twitch muscle concerns the release and uptake of Ca^{2+} by the sarcoplasmic reticulum rather than changes in actomyosin ATPase activity, since changes in activation kinetics may be more important during single isometric twitches than during the cyclic contractions of tetanus, where cross-bridge kinetics are more likely to be rate limiting.

HPX resulted in increased fatigue of the two fast-twitch muscles (MG and FDL), but not of the soleus. This may have been a result of greater decrease of activity of the metabolic

enzymes of the fast oxidative glycolytic and fast glycolytic fibres in the presence of low thyroid hormone concentrations (Baldwin, Hooker, Campbell & Lewis, 1978). Also, there may have been a decrease in the capillarity of the muscles after HPX because it has been shown that HPX of pig fetuses on day 45 of gestation produced a marked reduction in capillary to fibre ratio in the semitendinosus muscle (Hausman, 1989). This retardation of blood vessel development may have a profound effect on the oxidative capacity of a muscle.

HPX has also been shown to decrease circulating levels of insulin-like growth factor I (IGF-I) in fetal sheep, without change of IGF-II levels (Mesiano, Young, Hey, Browne & Thorburn, 1989). IGF-I stimulates proliferation and differentiation of myoblasts and satellite cells (Allen & Boxhorn, 1989) and IGF-I mRNA levels increase in muscle undergoing compensatory or stretch-induced hypertrophy (Czerwinski, Martin & Bechtel, 1994). HPX resulted in decreased levels of IGF-I in semitendinosus muscle of fetal pigs (Latimer, Hausman, McCusker & Buonomo, 1993). Although the IGF levels in the muscles of HPX sheep fetuses are not known it is possible that some of the effects observed in our study, in particular, the reduced growth of the soleus muscle, may result from reduced levels of this growth factor. Also, in cultures of L6 muscle cells, it has been shown that IGF-I potentiates the stimulatory effect of thyroid hormone on Ca^{2+}-ATPase activity of the sarcoplasmic reticulum (Muller, van Hardeveld, Simonides & van Rijn, 1991). Thus, the prolongation of the twitch profile of the MG and FDL muscles in the HPX fetuses may be due to changes of the Ca^{2+} kinetics which result from the combined effects of a decreased availability of IGF-I and thyroid hormone.

In conclusion, we have shown that the differentiation of fast- and slow-twitch characteristics of the hindlimb muscles of fetal sheep occurs between 90 and 125 days gestation in sheep. It is noteworthy that these changes differ in sequence from that described in altricial species such as the cat in at least one respect. In kittens, the fast- and slow-twitch hindlimb muscles have similar twitch contraction and relaxation times at birth. The differentiation which occurs in the next 6–10 postnatal weeks involves a decrease of the twitch contraction times of both fast and slow muscles, but with a significantly greater decrease occurring in fast-twitch muscles (Buller *et al.* 1960). This is different from the changes reported in this study, where there was a decrease of the contraction time in fast-twitch muscles, but a prolongation of contraction time in the slow-twitch soleus muscle. The results of this study also show that the two 'fast-twitch' muscles are dependent, to some extent, upon pituitary factors for their functional development, whereas the soleus muscle requires pituitary control for its growth, but not its functional development at the end of gestation.

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